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An Internal Standard Method for the Unattended High-Performance Liquid Chromatographic Analysis of Ascorbic Acid in Blood Components[1]

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A paired-ion, reversed-phase, high-performance liquid chromatography procedure using electrochemical detection and internal standard quantitation based on isoascorbic acid (IA) is described for the determination of ascorbic acid (AA) in blood cells and plasma. By correcting for vial-to-vial variations in the AA oxidation rate, use of IA as an internal standard overcomes a major problem associated with AA instability and eliminates the necessity of assaying samples immediately after they are prepared for analysis. The ion-pairing agent, dodecyltriethylammonium phosphate, gives improved AA–IA resolution over agents with shorter carbon chains and also eliminates the interference of an unidentified substance extracted with platelet AA. Five percent metaphosphoric acid extracts of mononuclear leukocytes (MN), polymorphonuclear leukocytes (PMN), platelets, or plasma were mixed with the IA internal standard and diluted with an EDTA–cysteine solution. The samples were placed in a refrigerated autosampler at 4°C prior to chromatography on a 5-μm octadecylsilyl column. AA concentrations (mean ± SD) in platelets, MN, and PMN from six healthy volunteers were 0.25 ± 0.05, 15.2 ± 6.28, and 2.43 ± 1.63 $\mu g/10^8$ cells, respectively; the mean plasma AA concentration was 0.97 ± 0.34 mg/dl. All are in good agreement with published values. Refrigerated sample extracts containing the internal standard can be reassayed up to 3 weeks later with negligible change in calculated AA concentration. Up to 70 samples can be assayed per day with a detection limit (3 × SD) and minimum quantifiable level (less than 5% coefficient of variation) of 0.02 and 0.2 ng/injection, respectively.

KEY WORDS: HPLC techniques; vitamins; ascorbic acid; leukocytes; platelets; blood plasma.

Paired-ion, reversed-phase, high-performance liquid chromatography coupled with electrochemical detection is a selective and sensitive technique for determining AA[3] in biological samples, foods, and pharmaceuticals. However, the strongly acidic conditions required to preserve AA in its reduced form during extraction, storage, and analysis can interfere with this type of chromatography (1). For example, MPA, an effective and widely used AA extractant and stabilizer (2), precipitates with many ion-pairing agents and can cause high column back-pressures or extraneous chromatographic peaks (3–5).

Dilution of the acid reduces these effects, but leaves AA vulnerable to oxidation unless it is assayed quickly. Furthermore, vial-to-vial variability in the AA oxidation rate becomes extreme when diluted samples are stored for several hours in a refrigerated autosampler while awaiting analysis. Addition of metal chelators, oxygen scavengers, or reducing agents does not prevent significant and highly variable oxidation of AA.

An internal standard which closely resembles AA in both its electrochemical properties and its stability could correct for vial-to-

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[3] Abbreviations used: AA, ascorbic acid; MPA, metaphosphoric acid; IA, isoascorbic acid; CysH, cysteine; MN, mononuclear leukocytes; PMN, polymorphonuclear leukocytes; UA, uric acid; ODS, octadecylsilyl.

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vial variability in both oxidation rate and injection volume as well as serve as a recovery marker for AA extraction. Using such an approach, large numbers of samples could be prepared at one time and loaded into an HPLC autosampler for unattended analysis over several hours.

IA has proven to be a suitable internal standard in this regard. When assayed by the paired-ion, reversed-phase HPLC method reported here, diluted human blood cell and plasma extracts show negligible change in calculated AA concentration based upon the AA/IA peak area ratio after 24 h at room temperature or 3 weeks at 4°C. The ion-pairing agent, dodecyltriethylammonium phosphate, provides excellent resolution of the two isomers and is compatible with MPA. Up to 70 samples can be assayed per day with a detection limit (3× SD at the lowest level tested) of 0.02 ng/injection and a minimum quantifiable level (with less than 5% coefficient of variation) of 0.2 ng/injection.

MATERIALS AND METHODS

Standard and sample preparation. Standards were prepared from frozen stock solutions containing 1 mg/ml AA or IA in 5% (w/v) MPA containing 0.54 mM Na_2EDTA. Stock solutions were diluted to produce working standards containing 10 to 400 ng AA and 200 ng IA/ml in 0.2% (w/v) MPA, 0.54 mM Na_2EDTA, and 1.0 mM CysH. Stock solutions are stable for several months at −70°C.

Duplicate whole blood samples from healthy volunteers were separated into MN, PMN, and platelets by centrifugation in a discontinuous Percoll gradient according to the method of Omaye *et al.* (6). AA from the isolated cell fractions was extracted into cold 5% (w/v) MPA containing 0.54 mM Na_2EDTA and stored at −70°C until analysis. Plasma was mixed with an equal volume of cold 10% (w/v) MPA containing 0.54 mM Na_2EDTA and centrifuged to remove the precipitated plasma proteins, and the supernatant was stored at −70°C until analysis. Upon thawing, samples were mixed with an equal volume of cold 5% (w/v) MPA containing 0.54 mM Na_2EDTA and 10 μg/ml IA. The resulting sample solutions were diluted 25-fold with cold 1.04 mM CysH containing 0.54 mM Na_2EDTA.

All samples and standards were kept at 4°C during preparation. They were filtered through disposable 0.2-μm filters (Gelman Sciences, Ann Arbor, MI) into glass HPLC vials and capped. While in the autosampler, they were maintained at 4°C. The injection volume was 50 μl. Samples and standards were prepared so that the range of sample AA concentrations was bracketed by the range of standard AA concentrations. A least-squares linear regression calibration plot of standard AA/IA peak area ratios versus AA/IA concentration ratios was then used to determine sample AA concentrations based on the sample AA/IA peak area ratios. All sample concentrations are reported as means ± SD.

Ascorbate oxidase treatment. Fifty-microliter extracts of plasma, MN, PMN, and platelets in 5% (w/v) MPA were diluted to 2.5 ml with cold 0.066 M sodium phosphate buffer, pH 6.5. The resultant pH of the diluted extracts was 5.4. Half of each diluted extract was filtered and immediately assayed for AA. The other half was incubated for 25 min at room temperature with 2.5 units of ascorbate oxidase (EC 1.10.3.3, Sigma Chemical Co., St. Louis, MO), filtered, and assayed for AA (7).

Precision. Within-assay reproducibility of the standard AA/IA peak area ratios was estimated by assaying one freshly prepared set of multilevel calibration standards six times in 1 day. Total between-assay standard reproducibility was estimated by preparing and assaying a multilevel standard curve once a day for 6 days. All standards were prepared from the same frozen AA and IA stock solutions.

To estimate sample reproducibility, frozen 5% MPA extracts of plasma, MN, PMN, and platelets were thawed and diluted as de-

scribed above. A set of calibration standards was also prepared for each sample set. The samples and their respective standards were chromatographed twice on the day of preparation: once immediately after dilution in order to determine initial concentrations, and again after 10 h storage in the autosampler at 4°C. The diluted samples with their standards were chromatographed again after 21 days storage at 4°C in the dark, and again after storage at room temperature in the dark for 24 h. Sample concentrations were recalculated after each storage interval and compared with the initial concentrations by a two-tailed, paired t test at the 0.05 level of significance.

Chromatography. The chromatography system (Perkin-Elmer, Norwalk, CT) included a Series 4 solvent delivery system, an LCI 100 integrator, an ISS 100 refrigerated autosampler, and a Model 7500 computer for data processing. It was fitted with a 250 × 4.6-mm Altex Ultrasphere ODS column and a 30 × 4.6-mm Brownlee RP-18 guard column (Rainin Instrument Co., Woburn, MA). Both columns contained spherical 5-μm particles.

The mobile phase was 40 mM sodium acetate, 0.54 mM Na_2EDTA, 1.5 mM dodecyltriethylammonium phosphate (Regis Chemical Co., Morton Grove, IL), and 7.5% methanol, taken to pH 4.75 with glacial acetic acid. It was filtered through a 0.2-μm nylon filter (Rainin Instrument Co., Woburn, MA) prior to use and degassed with a helium sparging system. Elution was isocratic at a flow rate of 0.8 ml/min and at ambient temperature. The detection system (Bioanalytical Systems, Inc., West Lafayette, IN) consisted of an LC4B amperometric controller and a thin-layer electrode cell containing a glassy-carbon working electrode, a stainless-steel electrode top, and an Ag/AgCl reference electrode. The applied potential was +0.5 V(oxidative) with a sensitivity setting of 50 nA.

Both column and electrode required about 6 h equilibration after beginning mobile phase flow. During this period several injections of an AA/IA standard were made until a constant background current (typically about 0.5 nA) and stable retention times (14.8 and 16.1 min for AA and IA, respectively) were achieved. Between batches of samples, the flow was reduced to 0.1 ml/min to conserve mobile phase. At the end of the week, the column was washed with 50 to 100 ml water followed by 50 to 100 ml methanol and stored in methanol over the weekend.

RESULTS

Identity and Specificity

Retention time correspondence of sample and standard AA and coelution of added standard AA with endogenous AA demonstrated identity. Incubation of representative plasma, MN, PMN, and platelet extracts with ascorbate oxidase caused complete loss of the AA peak as determined by HPLC analysis before and after enzyme treatment. These results confirmed the identity of the AA peak and established the absence of coeluting substances in these samples. In addition, six other oxidizable and electrochemically active biological substances did not interfere with the AA or IA peaks. Epinephrine, norepinephrine, CysH, and homocysteine were eluted in the void volume or very shortly thereafter. Glutathione and UA were eluted as separate peaks about midway between the void volume and the ascorbate isomers. Representative chromatograms of diluted plasma, MN, PMN, and platelet extracts are shown in Fig. 1. All had peaks for added CysH and IA in addition to the endogenous AA. The plasma chromatogram contained a large UA peak which can also be quantified. The platelet extracts contained a substance(s) which was eluted in a broad, asymmetric peak after CysH. This peak was also seen in chromatograms of cell extracts which had significant platelet contamination.

Standard Calibration Curve

The standard calibration curve was linear over a range of 0.2 to at least 30 ng AA in

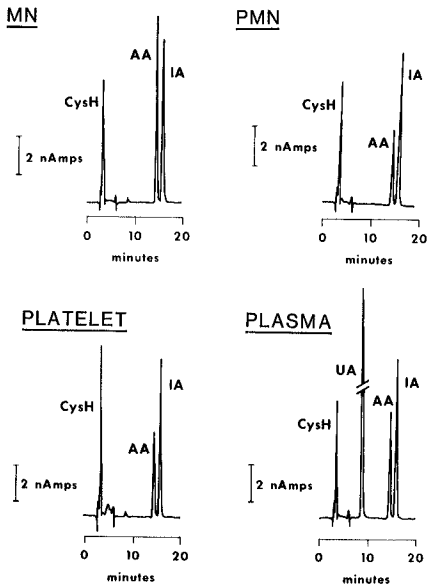

FIG. 1. Chromatograms of 50-μl diluted cell extracts and plasma containing 10 ng IA in 0.2% MPA, 0.54 mM Na_2EDTA, and 1 mM CysH: MN (10.8 ng AA), PMN (4.8 ng AA), platelets (5.2 ng AA), and plasma (6.5 ng AA). Chromatographic conditions were as described under Materials and Methods.

the presence of 10 ng IA per injection ($r > 0.999$). Outside this range, the electrode response became nonlinear. The detection limit (3× SD of the lowest standard tested) was 0.02 ng AA per injection and the minimum quantifiable level (with coefficient of variation < 5%) was 0.2 ng AA per injection. In a typical standard curve, AA ranged from 0.5 to 20 ng in the presence of 10 ng IA per injection. Within-assay and total between-assay precision for AA/IA peak area ratios of six standard levels in this range are shown in Table 1. Within-assay and total between-assay coefficients of variation were 3.1% or less and 6.7% or less, respectively.

AA Stability in Standard Solutions

Figure 2 shows the effect of storage at 4°C over a 16-h interval on AA peak areas and AA/IA peak area ratios for a set of multilevel standard solutions. After 16 h, AA peak areas had decreased to 70 ± 27% of their initial values, while the AA/IA peak area ratios remained at 100 ± 4% of their initial values. Because AA/IA peak area ratios were constant over this time interval and vial-to-vial variations were negligible, all quantitation was based upon AA/IA peak area ratio.

TABLE 1

VARIABILITY IN CALIBRATION STANDARD AA/IA PEAK AREA RATIOS

ng AA injected	Coefficient of variation (%)	
	Within-assay	Total between-assay
0.5	3.1[a]	6.7
1.0	1.9	2.3
2.0	0.6	1.4
5.0	0.3	1.4
10.0	0.6	1.8
20.0	0.1	1.7

Note. Fifty-microliter injections containing 10 ng IA in 0.2% MPA, 0.54 mM Na_2EDTA, and 1.0 mM CysH.
[a] $n = 5$; for all others $n = 6$.

Sample Stability and Reproducibility

Table 2 shows the cumulative effect of three sequential storage intervals on the initial calculated AA concentrations (based upon the AA/IA peak area ratio) for diluted plasma, platelet, MN, and PMN extracts.

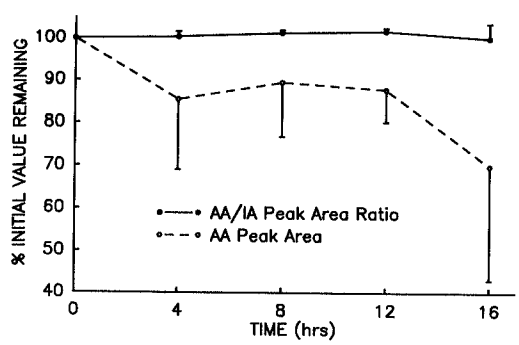

FIG. 2. Effect of storage at 4°C on AA peak area and AA/IA peak area ratio for standard AA solutions. Each point is the mean with SD for four standards containing 2 ng IA and 1, 2, 5, or 10 ng AA/50-μl injection. Chromatographic conditions were as described under Materials and Methods.

There were no significant changes for any sample type during the first 10 h at 4°C. Some small, but statistically significant, increases in the calculated AA concentration were observed in plasma, platelet, and MN extracts after 21 days at 4°C or 1 day at room temperature. All other changes in calculated AA concentrations were not significant. The small standard deviations associated with calculated AA concentrations in all sample types over the three intervals demonstrate that variations in the AA/IA peak area ratio are negligible during storage under these conditions.

Table 2 also shows the cumulative effect of the three storage intervals on AA peak area for the four sample types. In general, large decreases in AA peak area were observed after the second and third storage intervals. For all 46 diluted sample extracts in this table, the mean percentage of initial AA peak area remaining after the third interval was 65 ± 15%, while the mean calculated AA concentration was 101 ± 4% of the initial value.

Sample AA Concentrations

The mean calculated AA concentrations (±SD) in platelets, MN, and PMN isolated from the blood of six healthy volunteers were 0.25 ± 0.05, 15.2 ± 6.28, and 2.43 ± 1.63 $\mu g/10^8$ cells, respectively. The corresponding plasma concentration was 0.97 ± 0.34 mg/dl. These values are all in good agreement with published values (8,9).

DISCUSSION

Structurally, IA differs from AA only in the orientation of the C-5 hydrogen and hydroxyl group; however, IA has only 5% of the vitamin activity of AA (10). It is very rarely found in nature, principally in certain species of the genus *Penicillium* (11). Its major

TABLE 2

CUMULATIVE EFFECT OF THREE SEQUENTIAL STORAGE INTERVALS ON CALCULATED AA CONCENTRATION AND AA PEAK AREA FOR DILUTED SAMPLE EXTRACTS[a]

	Percentage of initial value remaining after storage interval					
	10 h at 4°C		21 days at 4°C		1 day at room temperature	
	Calcd concn	Peak area	Calcd concn	Peak area	Calcd concn	Peak area
Plasma (n = 12)	100 ± 1	100 ± 7	103 ± 1 ($P < 0.0001$)[b]	69 ± 11	103 ± 2 ($P = 0.0001$)[b]	59 ± 13
MN (n = 12)	99 ± 2	92 ± 21	99 ± 2	81 ± 18	103 ± 3 ($P = 0.0013$)[b]	73 ± 17
PMN (n = 10)	99 ± 1	97 ± 3	101 ± 2	71 ± 18	100 ± 7	59 ± 18
Platelets (n = 12)	100 ± 1	97 ± 8	101 ± 1 ($P = 0.0121$)[b]	74 ± 5	100 ± 1	67 ± 7

[a] Plasma and cell extracts from duplicate blood samples taken from six individuals (five for PMN) were prepared as described under Materials and Methods. Initial AA concentrations were determined and then all samples and standards were stored sequentially for 10 h at 4°C, followed by 21 days at 4°C, followed by 24 h at room temperature. Samples and standards were reassayed at the end of each storage interval and the AA concentrations were determined from the AA/IA peak area ratios. Percentage calculated AA concentration and percentage AA peak area remaining were calculated from the following expression: (value at end of storage interval)/(initial value) × 100. All values shown are the mean of 10 or 12 determinations ± SD.

[b] P value for significant cumulative change from the initial calculated AA concentration after the storage interval determined by a two-tailed paired t test at the 0.05 level. All other changes from the initial calculated AA concentration were not significant.

uses are as an antioxidant and a curing agent in meats.

It is unlikely that any significant tissue IA levels would ever be encountered in free-living humans. In a controlled study (H. E. Sauberlich, J. H. Skala, S. T. Omaye, and M. A. Kutnink, unpublished work), six volunteers ingesting 600 mg IA daily achieved and maintained plasma IA levels of 0.30 ± 0.05 mg/dl. Extrapolating from this value and total ascorbate levels (AA + IA) measured in cured meats (12), one would have to ingest at least 770 g (1.7 lb) per day of uncooked ham or frankfurters cured with IA in order to achieve a plasma IA level sufficient to cause a 5% increase in the internal standard concentration. We have analyzed plasma and blood cells from 34 free-living individuals by HPLC methods capable of detecting IA and have found none.

At 25°C, IA oxidizes faster than AA between pH 3 and pH 7 in the presence of Cu^{2+} (13), the rates for both isomers increasing with pH. Our diluted samples and standards contained 0.2% MPA, 0.54 mM NA_2EDTA, and 1 mM CysH at pH 2.7. Both EDTA and CysH decrease the oxidative effects of Cu^{2+} and Fe^{2+} by chelation. CysH also removes dissolved O_2 (14). Although some of the changes in calculated AA concentration shown in Table 2 are statistically significant, they are of a magnitude comparable to the between-assay variance and would be of no practical significance in typical applications when the samples would be stored refrigerated for only a few days. The stability of the calculated sample AA concentrations during storage at room temperature or with refrigeration demonstrates that the difference between AA and IA oxidation rates in these sample matrices is negligible. Quantitation based upon the AA/IA peak area ratio compensates for large losses of the ascorbate isomers and for vial-to-vial variations in the oxidation rate.

While not investigated in this study, the IA internal standard method could prove to be even more useful if the IA were added to fresh samples with the MPA/EDTA extractant prior to freezing. Such an approach might correct for variations in AA extraction and AA degradation during long-term freezer storage. Further work is needed to determine whether these advantages can be realized.

The ion-pairing agent in this method, dodecyltriethylammonium phosphate, does not form precipitates with MPA. We have experienced no high back-pressures or interfering peaks which are sometimes encountered when other cationic ion-pairing agents are used with MPA-stabilized samples. Dodecyltriethylammonium phosphate also gives improved resolution of AA and IA over ion-pairing agents with shorter carbon chains, and eliminates the interference of a broad, asymmetric peak seen in chromatograms of platelet extracts (retention time = 4 min, Fig. 1). This peak caused major interference with platelet AA determinations when tetrabutylammonium phosphate was the ion-pairing agent (M. A. Kutnink, unpublished observation).

UA is well-resolved by this procedure, and, because it oxidizes at the same electrode potential as the ascorbate isomers, it can be quantified in the same sample used for AA analysis. Unlike AA, UA is quite stable in diluted sample and standard solutions and can be adequately quantified using external UA calibration standards. We have quantified UA in eight commercially available lyophilized normal and elevated control plasmas using this chromatographic procedure. The values obtained were within the expected ranges published with these calibrated control plasmas.

As with many paired-ion methods, there is some retention time drift. Both AA and IA retention times and the resolution between them decrease slightly as the number of injections increases. Retention times and resolution are completely restored by washing the column with about 50 ml water followed by 50 ml methanol. The decrease in resolution appears to be linearly related to the number of injections ($r^2 = 0.955$). The resolution between AA and IA, based on peak

width at base, of a freshly washed column is 2.25. Assuming a minimum acceptable resolution of 1.0 (90% complete separation), which is considered adequate for peak area quantitation (15), about 200 sample or standard injections can be made between methanol washes. This is equivalent to about 67 h of uninterrupted operation at a sampling rate of 3 per hour.

The use of IA as an internal standard for quantitation of AA in the HPLC method presented here overcomes many of the problems associated with AA instability while maintaining the selectivity and sensitivity of electrochemical detection. Because the isomers oxidize at essentially the same rate in refrigerated working standard solutions and diluted blood cell or plasma extracts, they can be reassayed after several days storage with negligible change in calculated AA concentration. Many samples can be loaded into an automated HPLC system for unattended analysis. The procedure may also lend itself to complete automation when coupled with an automated extraction procedure similar to the one recently described by Vanderslice and Higgs (16).

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